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Sundquist, Elias

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Tenascin-C and fibronectin expression divide early stage tongue cancer into low- and high-risk groups

Elias Sundquist^{*,1,2}, Joonas H Kauppila^{1,2,3}, Johanna Veijola^{4,5}, Rayan Mroueh⁶, Petri Lehenkari^{1,2}, Saara Laitinen⁷, Juha Risteli^{2,8,9}, Ylermi Soini^{10,11}, Veli-Matti Kosma^{10,11,12}, Iris Sawazaki-Calone¹³, Carolina Carneiro Soares Macedo¹⁴, Risto Bloigu¹⁵, Ricardo D Coletta¹⁴ and Tuula Salo^{1,2,16}

¹Cancer and Translational Medicine Research Unit, University of Oulu, Oulu FI-90014, Finland; ²Medical Research Centre, Oulu University Hospital, Oulu FI-90014, Finland; ³Department of Molecular Medicine and Surgery, Upper Gastrointestinal Surgery, Karolinska Institutet, Karolinska University Hospital, Stockholm 17176, Sweden; ⁴Department of Physiology, Institute of Biomedicine, University of Oulu, Oulu FI-90014, Finland; ⁵Department of Biochemistry and Molecular Medicine, University of Oulu and Biocenter Oulu, Oulu FI-90014, Finland; ⁶Department of Otorhinolaryngology—Head and Neck Surgery, University of Helsinki and Helsinki University Hospital, Helsinki FI-00014, Finland; ⁷Department of Research and Development, Finnish Red Cross Blood Service, Helsinki FI-00014, Finland; ⁸Department of Clinical Chemistry, Institute of Diagnostics, University of Oulu, Oulu FI-90014, Finland; ⁹Northern Laboratory Centre NordLab, Oulu FI-90220, Finland; ¹⁰Department of Clinical Pathology and Forensic Medicine, University of Eastern Finland, Kuopio FI-70211, Finland; ¹¹Cancer Centre of Eastern Finland and Kuopio University Hospital, Kuopio FI-70210, Finland; ¹²Department of Clinical Pathology, Imaging Centre, Kuopio University Hospital, Kuopio FI-70211, Finland; ¹³Oral Pathology and Oral Medicine, Dentistry School, Western Paraná State University, Cascavel 85819-110, Brazil; ¹⁴Department of Oral Diagnosis, Oral Pathology Division, Piracicaba Dental School, University of Campinas, Campinas 13414-903, Brazil; ¹⁵Medical Informatics and Statistics Research Group, University of Oulu, Oulu FI-90014, Finland and ¹⁶Department of Oral and Maxillofacial Diseases, University of Helsinki, Helsinki FI-00014, Finland

Background: Oral tongue squamous cell carcinoma (OTSCC) metastasises early, especially to regional lymph nodes. There is an ongoing debate on which early stage (T1-T2N0) patients should be treated with elective neck dissection. We need prognosticators for early stage tongue cancer.

Methods: Mice immunisation with human mesenchymal stromal cells resulted in production of antibodies against tenascin-C (TNC) and fibronectin (FN), which were used to stain 178 (98 early stage), oral tongue squamous cell carcinoma samples. Tenascin-C and FN expression in the stroma (negative, moderate or abundant) and tumour cells (negative or positive) were assessed. Similar staining was obtained using corresponding commercial antibodies.

Results: Expression of TNC and FN in the stroma, but not in the tumour cells, proved to be excellent prognosticators both in all stages and in early stage cases. Among early stages, when stromal TNC was negative, the 5-year survival rate was 88%. Correspondingly, when FN was negative, no cancer deaths were observed. Five-year survival rates for abundant expression of TNC and FN were 43% and 25%, respectively.

Conclusions: Stromal TNC and, especially, FN expressions differentiate patients into low- and high-risk groups. Surgery alone of early stage primary tumours might be adequate when stromal FN is negative. Aggressive treatments should be considered when both TNC and FN are abundant.

*Correspondence: Dr E Sundquist; E-mail: elias.sundquist@oulu.fi

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Globally an estimated 405 000 new cases of oral cancer are diagnosed annually, of which about 50% are located in the oral/mobile tongue. Oral tongue squamous cell carcinoma (OTSCC) is an aggressive subset of the oral squamous cell carcinomas (OSCCs) with the highest invasion and metastasis properties (Bello *et al*, 2011; Ganly *et al*, 2012; Kim and Cha, 2012). Lymph node metastases are the single most important prognostic factor of OSCC. However, in early stage cancers, lymph node metastases are often occult without preoperative signs (Bello *et al*, 2010). The prognosis can be improved by performing elective neck dissection for all early stage OTSCC patients (D'Cruz *et al*, 2015), but most of the patients do not benefit from elective neck dissection, from which surgery-related morbidities are substantial (Kelner *et al*, 2015). Owing to the lack of prognostic markers, the mortality of early stage (cT1-T2N0) OTSCC is still comparatively high (Ganly *et al*, 2012), as patients with aggressive OTSCC are not recognised. Thus the development of reliable methods to detect aggressive early stage tumours is essential in order to provide treatment with the most efficient modalities.

The role of the tumour microenvironment (TME) in cancer development has become abundantly clear. The fibroblasts and extracellular matrix (ECM) components are crucial for the proliferation and invasion of cancer cells through dynamic interactions between cancer cells and ECM molecules (Metwally *et al*, 2012). As mesenchymal stem cells, among other TME components, are known to enhance the invasion of OTSCC cells (Sobral *et al*, 2011; Salo *et al*, 2013), we used intact human mesenchymal stromal cells (hMSCs) as an immunising agent to produce specific antibodies in mice. The aim of this study was to characterise novel monoclonal mouse antibodies produced against hMSCs, and to evaluate the prognostic value of two of the target antigens – tenascin-C (TNC) and fibronectin (FN) – in OTSCCs according to the reporting recommendations for tumour marker prognostic studies (REMARK) criteria.

MATERIALS AND METHODS

New anti-TNC and anti-FN antibodies. Mice (C57BL/6) were immunised using cultured live hMSCs as single-cell suspensions in PBS (i.p.). The hMSCs were obtained from bone marrow (BM) of volunteer donors (two 16-year-old donors with arthritis) following written consent approved by the Ethical Committee of the Northern Ostrobothnia Hospital District, Oulu, Finland. Bone marrow hMSC isolation and culture followed the protocol described by Leskelä *et al* (2003). The presence of both MSC-positive (CD73, CD90 and CD105) and MSC-negative (CD14, CD19, CD34, CD45 and HLA-DR) cell surface markers of the cultured cells were assayed by flow cytometry, and the differentiation potential of isolated cells was assessed for adipogenic and osteogenic differentiation capability (data not shown). The preparation of the live cells and the booster immunisation was performed twice. On the basis of the antibody titres, mice were chosen and their spleens prepared for fusion. Positive hybridomas were subcloned several times using standard limiting dilution procedures.

Media from hybridoma lines were screened against several cell types by cyto-ELISA (Grunow *et al*, 1994), flow cytometry and western blot. The monoclonal antibodies produced by the hybridoma lines were isolated using protein G. The target antigens were isolated from cultured hMSC cell homogenate by immunoprecipitation with the purified antibody covalently coupled to the surface of Dynabeads (M270, Invitrogen, Carlsbad, CA, USA) or using Protein G resin (Sigma-Aldrich, Ayshire, UK). The immunoprecipitated target molecules were separated using SDS-7.5% PAGE. The bands from protein gels (Figure 1A and B) were identified using peptide fingerprinting (trypsin cleavage and MS Maldi TOF

analysis of proteolytic fragments) and Mascot Database search (Matrix Science, London, UK).

Purified antibodies were assayed for immunofluorescence using the Dylight 550 conjugated anti-mouse secondary antibody and DAPI. The formalin fixed, paraffin-embedded synovial tissues were also assayed with the new antibodies (Figure 1C). The negative control was stained with the idiotype antibody (unrelated mouse IgG1). For western blots of various cell types and tissues, the samples were incubated with the new matrix antibodies at a concentration of $1 \mu\text{g ml}^{-1}$ for 2 h and, after washing, the bound antibodies were detected with peroxidase conjugated anti-mouse antibody (Sigma-Aldrich). Target antigen for antibodies from two clones (A8 and D2) was identified as human TNC and target antigen for antibodies from three clones (F12, C1 and B6) was identified as human FN. From these antibodies, the ones from clones D2 for TNC and F12 for FN were chosen to be used in this study.

Patients and biological material. This retrospective multi-centre study included 178 OTSCC patients treated in University Hospitals

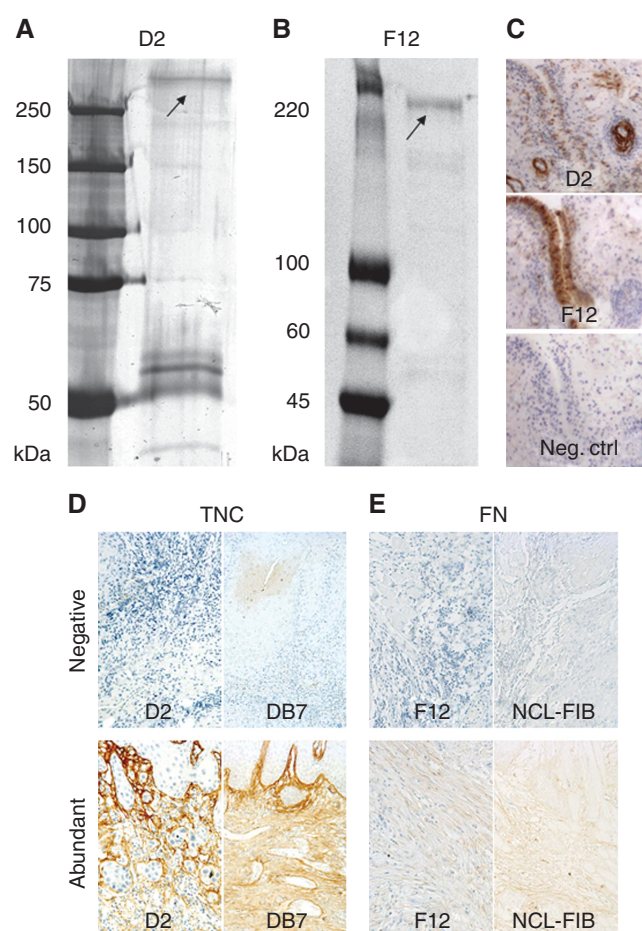


Figure 1. Characterisation of the new matrix antibodies. Coomassie-stained SDS-7.5% PAGE of the target antigens from the cultured hMSC lysate immunoprecipitated using the new anti-tenascin-C monoclonal antibody D2 (A) or anti-fibronectin monoclonal antibody F12 (B). Molecular weight markers are shown on the left of the gels: Bio-Rad Dual Colour (A) and Sigma Colorburst (B). The identified target antigen is marked with arrows. Bands migrating at ~ 60 kDa region are mouse antibody fragments detached from the resin (A). The immunostaining of the synovial tissue sections with anti-tenascin-C D2, anti-fibronectin F12 and idiotype IgG1 as negative control (C). New antibodies were compared with commercial ones by staining representative samples from 'negative' and 'abundant' groups using both D2 and commercial DB7 for TNC (D), and both F12 and commercial NCL-FIB for FN (E).

of Oulu (OUH, $n = 107$) and Kuopio (KUH, $n = 21$) in Finland from 1979 to 2009, and UOPECCAN Cancer Hospital ($n = 27$) and Oncology Center of Cascavel (CEONC, $n = 23$) in Brazil from 1998 to 2008. Of these 178 patients, 98 had been diagnosed with clinical stage I (cT1N0M0) or II (cT2N0M0) OTSCC. The inclusion criteria were sufficient clinical data and histological samples to perform the analysis. All patients had undergone primary resection of the tumour. Detailed patient demographic data of the early stage patients are shown in Table 1, and demographic data of the pooled group of all stage patients are in Supplementary Table 1. This study was approved by the Ethical Committee of the Northern Ostrobothnia Hospital District, Finland and the Finnish National Supervisory Authority for Welfare and Health. In Brazil, this study was approved by the Ethics Committee in Research of the Piracicaba Dental School, University of Campinas (approval number 100/2012).

Immunohistochemistry

TNC and FN. After characterisation, our novel TNC antibody D2 was qualitatively compared with the well-tested commercial monoclonal TNC antibody DB7 (order #610003, Biohit, Helsinki, Finland), and our FN antibody F12 was compared with the commercial monoclonal antibody NCL-FIB (NCL-FIB, Leica Biosystems, Newcastle, UK; Figure 1D and E). Representative OTSCC samples from negative and abundant groups were stained with both D2 and DB7, as well as F12 and NCL-FIB. Paraffin-embedded resected tumours were obtained and prepared for immunohistochemistry. In essence, 5 μ m thick slices were

mounted on microscope slides and dewaxed in xylene. For epitope retrieval, the sections were then heated in a microwave oven in Tris-EDTA solution (pH 9) for 10 min and allowed to cool at room temperature (RT) for 20 min. Endogenous peroxidase activity was blocked using peroxidase-blocking solution (DAKO, Glostrup, Denmark) for 10 min. The sections were then incubated with primary antibodies at a dilution of 1:500 for 60 min at RT. For visualisation, horseradish peroxidase (HRP)-conjugated antibodies (DAKO) were introduced to sections for 10 min, after which DAB chromogen was used for 5 min, and the sections were then counterstained using Mayer's haematoxylin.

α -Smooth muscle actin. A group of 60 randomly selected OTSCC samples were stained for α -smooth muscle actin (α -SMA). The sections were incubated with primary antibody (Dako monoclonal mouse anti-human, 1:500, clone 1A4, M0851) for 30 min in RT.

Analysis of TNC and FN in the stroma and tumour cells.

Stromal TNC and FN stain was scored as previously described for α -SMA (Bello *et al*, 2011), using a scale from 0 to 4 as follows: 0 = no extracellular staining; 1 = staining of individual confined spots; 2 = staining not confined to specific spots, more diffuse; 3 = staining throughout the invasive front, distinguishable border between the stained ECM and carcinoma cells; 4 = staining throughout the invasive front, no distinguishable border between the stained ECM and carcinoma cells. For statistical analysis the grade 0 was labelled as 'negative' (Figure 2A and E), grades 1–3 were merged into the 'moderate' group (Figure 2B and F) and

Table 1. Patient data of stage I and II OTSCC patients

N (%)	All patients (n = 98)	OUH (n = 59)	KUH (n = 14)	UOPECCAN (n = 8)	CEONC (n = 17)
Patient data, stages I–II					
Gender					
Male	53 (54)	24 (41)	9 (64)	6 (75)	14 (82)
Female	45 (46)	35 (59)	5 (36)	2 (25)	3 (18)
Age at diagnosis					
≤55	26 (26)	13 (22)	3 (21)	3 (38)	7 (41)
56–69	33 (34)	15 (25)	7 (50)	4 (50)	7 (41)
≥70	39 (40)	31 (53)	4 (29)	1 (12)	3 (18)
Range	32–99	32–99	33–80	45–77	40–80
Mean	64.18	67.17	62.29	56.50	59
Median	64	70	63.5	57	59
Follow-up time					
Range	1–298	1–298	3–175	6–127	1–139
Mean	73.65	80.75	72.71	61.38	55.59
Median	57	64	60	47.5	51
Cause of death					
Alive	49 (50)	31 (53)	6 (43)	5 (62)	7 (41)
Cancer	22 (22)	8 (14)	3 (21)	3 (38)	8 (47)
Other	27 (28)	20 (33)	5 (36)	–	2 (12)
Grade					
1	28 (28)	24 (41)	4 (29)	–	–
2	37 (38)	32 (54)	5 (36)	–	–
3	6 (6)	3 (5)	3 (21)	–	–
Missing	27 (28)	–	2 (14)	8 (100)	17 (100)
Neck lymph nodes					
Positive	2 (2)	2 (3)	–	–	–
Negative	96 (98)	57 (97)	14 (100)	8 (100)	17 (100)
Adjuvant therapy					
No	67 (69)	47 (80)	7 (50)	3 (37)	10 (59)
Radiotherapy	22 (22)	9 (15)	7 (50)	2 (26)	4 (23)
Radio- and chemotherapy	7 (7)	1 (2)	–	3 (37)	3 (18)
Missing	2 (2)	2 (3)	–	–	–
Recurrence					
Yes	39 (40)	17 (29)	8 (57)	5 (62)	9 (53)
No	59 (60)	42 (71)	6 (43)	3 (38)	8 (47)

Abbreviations: CEONC = Oncology Center of Cascavel; KUH = Kuopio University Hospital; OTSCC = oral tongue squamous cell carcinoma; OUH = Oulu University Hospital. Data as combination from all the hospitals, as well as separately from each hospital.

grade 4 was labelled as ‘abundant’ (Figure 2C and G). If the same tumour had areas of different staining intensity, the tumour was scored according to the most severe grade.

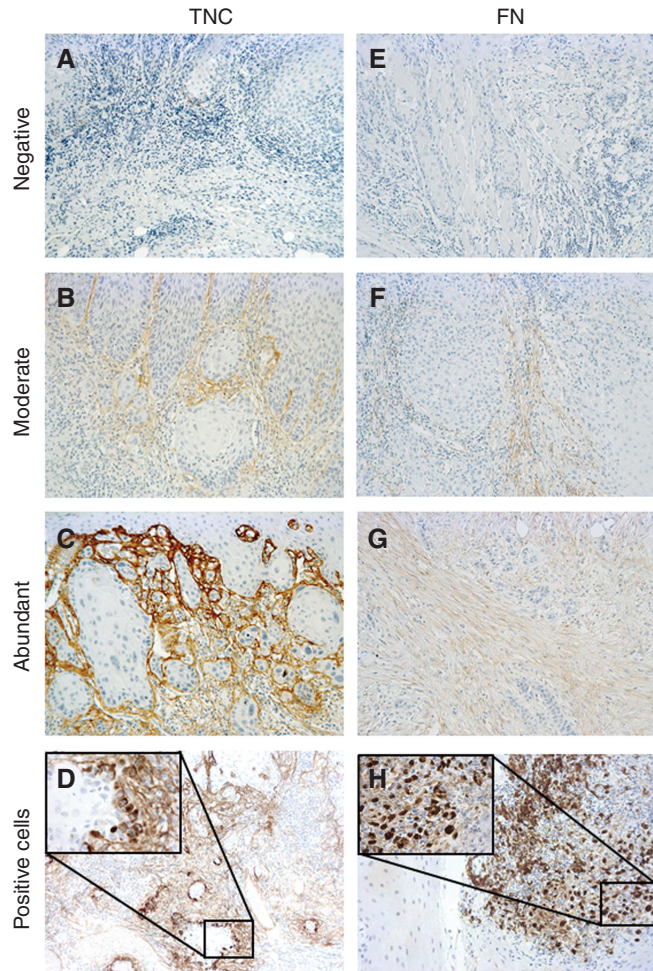


Figure 2. Scoring of stromal and intracellular TNC and FN. Stromal TNC (A–C) and FN (E–G) were scored from 0 to 4 and grade 0 was labelled ‘negative’ (A and E), grades 1–3 were combined and labelled ‘moderate’ (B and F) and grade 4 was labelled ‘abundant’ (C and G). Intracellular TNC (D) and FN (H) were scored from 0 to 2. Grade 0 was labelled ‘negative’ and 1–2 were combined and labelled ‘positive’ (D and H).

Expression in the carcinoma cells was scored as follows: 0 = no cells with intracellular staining; 1 = individual cells with intracellular staining and 2 = groups of cells with intracellular staining. For statistical analysis, groups 1 and 2 were merged into a ‘positive’ group (Figure 2D and H). Scoring was performed by two of the authors (ES or RM and JHK) independently and unaware of the clinical outcome at the time of the analysis. Discrepancies in the scoring were settled by an experienced oral pathologist (TS). Most of the discrepancies were related to scores 1–3; scores which were combined to the same group for statistical analyses. There were a total of 44 cases that were scored differently by the assessors due to stromal TNC in the pooled data of all the stages of disease. Of these cases, 17 would have been included in different groups. Furthermore, in 9 cases one of the assessors scored the sample as negative and the other one as ‘not-negative’. The respective numbers of cases for stromal FN scoring were 37, 16 and 8. Among early stage cases, the numbers were 25, 8 and 5 for TNC and 21, 8 and 6 for FN.

Statistical analysis. Survival curves were created using the Kaplan–Meier method with disease-specific death as the end point, and death by other cause and end of follow-up as censoring events. Differences between the groups were analysed by a log-rank test.

The prognosis of the patients related to stromal and intracellular TNC, and FN were evaluated by univariate analysis using the Cox proportional hazard model. Differences between the groups were evaluated using a χ^2 -test.

Multivariate analysis was conducted using the Cox proportional hazard model. In multivariate analysis the results were adjusted for age, gender, adjuvant therapy and hospital, as well as stage among the pooled group including all the stages. As there were no end points (cancer deaths) in the FN-negative group among patients with stage I–II OTSCC, no hazard ratios (HRs) could be calculated in reference to this group. Hence, HRs for stromal FN in the stage I–II patient group were calculated comparing ‘abundant’ with ‘moderate’.

Correlations between stromal TNC, FN and α -SMA were calculated using a Spearman’s correlation test and 2-tailed significance was applied. Kappa values for inter-rater agreement in TNC and FN scoring was performed by cross tabulation and statistical significance confirmed using a χ^2 -test. Cross tabulation was used for the analysis of association between clinical parameters and stromal TNC and FN staining.

In all the analyses P -values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using either

Table 2. Results of scoring of stromal and intracellular TNC, and FN of stage I and II OTSCC patients					
N (%)	All patients (n = 98)	OUH (n = 59)	KUH (n = 14)	UOPECCAN (n = 8)	CEONC (n = 17)
Frequencies of TNC and FN scores in different hospitals, stages I–II					
Stromal TNC					
Negative	26 (27)	20 (34)	–	2 (25)	4 (23)
Moderate	65 (66)	35 (59)	11 (79)	6 (75)	13 (77)
Abundant	7 (7)	4 (7)	3 (21)	–	–
Cellular TNC					
Negative	60 (61)	32 (54)	12 (86)	4 (50)	12 (71)
Positive	38 (39)	27 (46)	2 (14)	4 (50)	5 (29)
Stromal FN					
Negative	35 (36)	27 (46)	3 (21)	1 (13)	4 (24)
Moderate	55 (56)	28 (47)	10 (72)	6 (74)	11 (64)
Abundant	8 (8)	4 (7)	1 (7)	1 (13)	2 (12)
Cellular FN					
Negative	63 (64)	47 (80)	7 (50)	4 (50)	5 (29)
Positive	35 (36)	12 (20)	7 (50)	4 (50)	12 (71)

Abbreviations: CEONC = Oncology Center of Cascavel; FN = fibronectin; KUH = Kuopio University Hospital; OTSCC = oral tongue squamous cell carcinoma; OUH = Oulu University Hospital; TNC = tenascin-C. Amounts of various scoring groups shown in total and for each individual hospital separately.

OriginPro 2015 (OriginLab, Northampton, MA, USA) or SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA: IBM Corp.).

RESULTS

Immunohistochemical validation of tenascin-C and fibronectin antibodies. The immunisation of mice with live BM hMSCs resulted in a series of antibodies (anti-CD73, chondroitin sulphate proteoglycan, FN, TNC, type VI collagen and CD13). However, after testing these in paraffin-embedded OTSCC tissue sections, the FN and TNC gave the most specific and strongest staining signals. Two of the clones were characterised as clones of anti-TNC and three as anti-FN, respectively. One clone of each (D2 for TNC and F12 for FN) was selected to be used in immunohistochemistry. When we compared our novel antibodies with commercially available antibodies, the staining patterns were similar in all of the tested samples (Figure 1D and E) and met the same scoring criteria. However, less unspecific staining was detected in the samples stained with D2 in comparison with DB7 (Figure 1D). The number of cases in each staining group from each of the hospitals is indicated in Table 2 for early stages and in Supplementary Table 2 for all stages. In early stage samples, the concordance for TNC and FN staining was high (kappa 0.827 for TNC and kappa 0.849 for FN, Supplementary Table 2).

Stromal TNC predicts outcome in OTSCC. The expression patterns of TNC were variable when comparing the stromal fibroblast-like cell and cancer cell components of the tumours. Stromal TNC expression was not associated with any of the clinical and demographic parameters (gender, age, hospital, stage, neck lymph nodes and adjuvant therapy, Supplementary Tables 3 and 4). A total of 65 cancer deaths occurred among combined group of all the stages including 178 patients during the whole follow-up period.

In the pooled group (all stages of disease), abundant stromal TNC served as a prognosticator in univariate analysis when compared with the negative group, with a HR and 95% confidence intervals (CI) of 3.21 (1.48–6.96; Supplementary Table 5). Five-year cumulative survival rates were 70%, 70% and 20% for the negative, moderate and abundant groups, respectively (Supplementary Figure 1A and E). The numbers of cancer deaths/total number of patients in a given group during the whole follow-up time in the negative, moderate and abundant groups were 14 out of 43, 39 out of 119 and 12 out of 16, respectively. When compared with the stromal TNC-negative group, the age, gender, hospital, adjuvant therapy and stage-adjusted HR and 95% CI in the multivariate analysis for abundant group was 2.48 (1.04–5.90). Tenascin-C expression in the tumour cells did not have statistically significant prognostic value (Supplementary Figure 1C and Supplementary Table 5).

Stromal and intracellular FN predict outcome in OTSCC. The stromal expression of FN in fibroblasts and the fibrillary network was associated with hospital, stage, lymph node metastasis and treatment modalities in the combined group of all stages (Supplementary Table 3). Among stages I–II, the stromal expression of FN was associated only with adjuvant therapy (Supplementary Table 4).

In the pooled group (all stages of disease), stromal FN was an excellent prognosticator (Supplementary Figure 1B and Supplementary Table 5) with 5-year cumulative survival rates being 89%, 62% and 22% for the negative, moderate and abundant groups, respectively (Supplementary Figure 1E). The number of cancer deaths/total number of patients during the whole follow-up time in the negative, moderate and abundant groups was 5 out of 51, 43 out of 105 and 17 out of 22, respectively. In this pooled

group, intracellular FN expression also predicted a poor prognosis (Supplementary Figure 1D and Supplementary Table 5) with a 5-year cumulative survival rate of 75% for the negative group and 54% for the positive group (Supplementary Figure 1E). In the univariate analysis including all stages, HRs for moderate and abundant stromal FN were 5.45 (2.16–13.77) and 13.58 (4.97–37.11), respectively (Supplementary Table 5). The HR for FN-positive cancer cells was 2.06 (1.25–3.38). In multivariate analysis the respective HRs were 5.33 (2.07–13.73) and 9.64 (3.34–27.83), and 2.08 (1.22–3.56) for FN-positive cancer cells (Supplementary Table 5).

Subgroup analysis of TNC and FN stainings. As staining patterns of both TNC and FN were strongly associated with patient outcome, we did a subgroup analysis in early stage OTSCC patients. Among early stage patients, there were a total of 22 events for 98 patients during the whole follow-up period.

There was no difference in outcome observed between stromal TNC-negative and moderate groups (Figure 3A and E) or between cellular TNC-negative and positive groups (Figure 3C and E) among early stage patients. However, the group with abundant stromal TNC differed significantly from those with negative or moderate staining with a 5-year survival of 43% (in comparison with 88% for negative and 82% for the moderate groups). The HRs were 5.18 (1.29–18.61) and 6.54 (1.20–35.75) in uni- and multivariate analyses in comparison with the negative group, respectively (Table 3). The number of cancer deaths during the whole follow-up time in the negative, moderate and abundant groups was 5 out of 26, 13 out of 65 and 4 out of 7, respectively. Cancer cell TNC expression had no statistically significant prognostic value (Figure 3C and E and Table 3).

Surprisingly, no cancer deaths of early stage patients were observed when stromal FN was negative. Thus, all of the cancer-specific deaths occurred in the moderately or abundantly stained groups. The numbers of cancer deaths during the whole follow-up time in the negative, moderate and abundant groups were 0 out of 35, 15 out of 55 and 7 out of 8, respectively. The 5-year cumulative survival rates were 100%, 76% and 25% for the negative, moderate and abundant groups, respectively (Figure 3B and E). As there were no disease-specific deaths in the stromal FN-negative group, the HR for the abundant group was calculated by comparing it with the moderate group. This resulted in HRs of 6.10 (2.36–15.75) and 5.91 (1.75–19.94) in uni- and multivariate analyses, respectively (Table 3).

Intracellular FN proved to be a significant prognostic factor in early stage cases in Kaplan–Meier analysis, but not in Cox regression uni- or multivariate analysis with cumulative 5-year survival rates of 85% and 72% for negative and positive groups, respectively (Figure 3D and E; Table 3).

Correlations of stromal TNC, FN and α -SMA. Carcinoma-associated fibroblasts (CAFs), which are identified by their intracellular α -SMA, are known predictors for poor prognosis in various malignancies (de Vlieghere *et al*, 2015), including OTSCC (Kellermann *et al*, 2007; Li *et al*, 2015; Bagordagis *et al*, 2016). However, CAFs are not prognostic indicators at early stage OTSCC (Almangush *et al*, 2014; Kelner *et al*, 2015), only in patient material of pooled stages, and at late stages when the variation in prognostic markers does not affect the choice of treatment so drastically, as more effective modalities are utilised in all cases. In this study we re-evaluated the prognostic significance of stromal α -SMA using a pilot group of 60 (with 17 cancer deaths) random samples from our extensive patient material. Indeed, in concordance to previous work of Almangush *et al* (2014) and Kelner *et al* (2015), in our pilot group the stromal α -SMA was found not to be predictive of patient outcome in early stage cases (Supplementary Figure 2). In addition, among early stage cases only a weak correlation was found between stromal α -SMA and TNC (0.395; 0.344 in all stages)

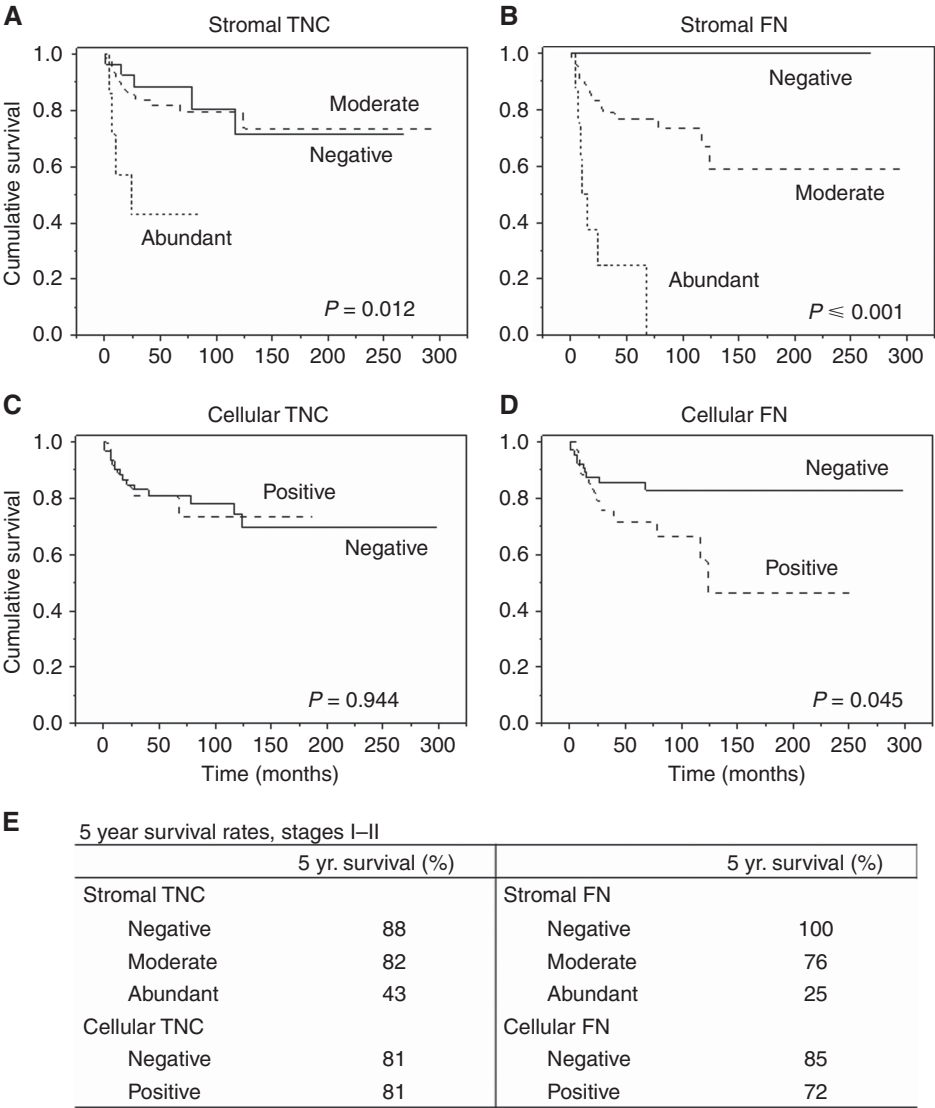


Figure 3. Survival of stage I and II OTSCC patients. Kaplan–Meier curves representing the cumulative survival of early stage OTSCC patients divided by stromal TNC (A) and FN (B) staining, as well as cellular TNC (C) and FN (D). In curves A–D the survivals are shown throughout the entire follow-up time. Five-year survivals are shown separately (E). Log-rank test was used to test statistical significance.

or FN (0.441; 0.402 in all stages, Supplementary Table 2), the two proteins shown in this study to have prognostic value in early stage OTSCC. Furthermore, a correlation was found in early stage cases between stromal TNC and FN with a correlation coefficient of 0.612 (0.617 in all stages, Supplementary Table 2).

After we found a strong correlation between stromal TNC and FN, we analysed those cases in which abundant staining of both these markers was observed in the stroma. A total of 14 patients showed abundant staining of both stromal TNC and FN, and 5 of these were among early stage OTSCC. Remarkably, among all stages there was only 1 patient alive after follow-up; 12 deaths were due to cancer and 1 due to another cause. There were no survivors at follow-up among the early stage patients; four were cancer deaths and one due to another cause.

DISCUSSION

There is a lack of potential markers to assess the need of elective neck dissection for early stage tongue cancer. In this international multi-centre study, we show that immunohistochemical staining

for FN and TNC could potentially be used in clinical decision-making for early stage OTSCC.

The strengths and limitations of our study should be taken into account when interpreting the results. Our study had an international, multi-centre study design, with which we aimed to counteract country-specific biases. The sample size in early stage group is small ($n=98$), especially in TNC and FN abundant groups, and might limit the statistical power and analyses in this group. This can be seen in the wide CIs in some of the analyses. However, the effects of TNC and FN expression were similar in both early and late-stage cancers, suggesting actual effect for the biomarkers. Finally, we adjusted for several pre-determined confounding factors in our analyses and showed that TNC and FN are associated to survival, also after the adjustment.

Although several OTSCC prognostic molecular markers have been reported (Bello *et al*, 2010), so far none is in clinical use to pinpoint those individuals who benefit from the primary neck dissection and multimodality treatment (Barnes *et al*, 2005). In this study, we hypothesised that the expression of two ECM proteins could predict the prognosis of cancers. After mice immunisation with stromal cells and production of related antibodies, we decided to assess the efficacy of TNC and FN as prognostic markers in

Table 3. Cox regression uni- and multivariate analyses of stage I and II OTSCC patients			
	HR	95% CI for HR	P-value
Univariate analysis, stages I–II			
Stromal TNC			0.025
Negative	1		
Moderate	1.09	0.39–3.07	NS
Abundant	5.18	1.29–18.61	0.019
Cancer cell TNC			NS
Negative	1		
Positive	1.03	0.43–2.48	NS
Stromal FN ^a			<0.001
Moderate	1		
Abundant	6.10	2.36–15.75	<0.001
Cancer cell FN			NS
Negative	1		
Positive	2.30	0.99–5.33	NS
Multivariate analysis, stages I–II			
Stromal TNC			0.012
Negative	1		
Moderate	0.76		NS
Abundant	6.54	0.23–2.48	0.030
Cancer cell TNC			NS
Negative	1		
Positive	1.61	0.54–4.78	NS
Stromal FN ^a			0.004
Moderate	1		
Abundant	5.91	1.75–19.94	0.004
Cancer cell FN			NS
Negative	1		
Positive	1.56	0.61–4.04	NS
Abbreviations: CI = confidence interval; FN = fibronectin; HR = hazard ratio; OTSCC = Oncology Center of Cascavel; TNC = tenascin-C. Multivariate analysis adjusted for age, gender and hospital. HR and their 95% CI, as well as P-values. Analyses were performed separately for stromal and cellular TNC and FN. In multivariate analysis the HRs were adjusted for age, gender and hospital. The HR for stromal FN in multivariate analysis was calculated by comparing abundant to moderate due to the lack of end points (cancer deaths) in the FN-negative group. Statistically significant values are bolded.			
^a HR could not be calculated comparing with 'Negative' group due to lack of end points in that group.			

OTSCC, as the role of these proteins as prognostic indicators in several cancers is well known (Jahkola *et al*, 1999; Emoto *et al*, 2001; Ioachim *et al*, 2002; Brunner *et al*, 2004; Ioachim *et al*, 2005; O'Shannessy *et al*, 2014). Neither of these proteins had been previously studied for their ability to predict prognosis of early stage OTSCC. In this study we showed that the identification of patients in low-risk and high-risk groups could potentially be achieved by assessing the stromal expression of TNC and FN. Stromal TNC and FN were both independent prognostic indicators not only in pooled stages of OTSCC, but most importantly in cases with clinical stages I–II when abundant stromal staining is detected.

Tenascin-C is an ECM glycoprotein with a six-armed quaternary structure. Under physiological conditions, TNC is found abundantly in several embryonic tissues, especially related to the epithelial mesenchymal transition (EMT), and to cell migration pathways. In adult tissues, it is expressed mainly during inflammation, wound healing and cancer progression (Orend and Chiquet-Ehrismann, 2006). Increased TNC expression in the tumour-adjacent ECM has been shown to correlate with a clinically aggressive phenotype or poor prognosis in several cancers such as oesophageal (Yang *et al*, 2016), breast (Ishihara *et al*, 1995; Jahkola *et al*, 1998) colorectal (Emoto *et al*, 2001; Sis *et al*, 2004), bladder (Brunner *et al*, 2004) and Merkel cell carcinomas (Koljonen *et al*, 2005); glioma (Herold-Mende *et al*, 2002), malignant pleural mesothelioma (Kaarteenaho-Wiik *et al*, 2003), glioblastoma multi-forme (Leins *et al*, 2003), malignant melanoma (Kääriäinen *et al*,

2006) and astrocytoma (Varga *et al*, 2012). In OSCC, TNC is produced by carcinoma cells (Driemel *et al*, 2007) or by surrounding mesenchymal fibroblast-like cells (Hindermann *et al*, 1999; Metwaly *et al*, 2012). Tenascin-C is present in the stroma of potentially malignant oral lesions and its expression is increased along the degree of dysplasia (Tiitta *et al*, 1994). Furthermore, TNC expression is high in budding cancer cells and in the surrounding stroma (Tiitta *et al*, 1994; Hindermann *et al*, 1999). Tenascin-C has a variety of pro-tumourigenic interactions with cancer cells, CAFs, lymphocytes and tumour-associated macrophages, as well as angiogenesis promoting interactions with endothelial cells. It interacts with cancer cells by reducing adhesion, enhancing proliferation, migration and invasion, and increasing dissemination and homing. Tenascin-C inhibits adhesion of CAFs, and activation, proliferation and cytokine production of tumour infiltrating lymphocytes (Midwood and Orend, 2009). Increased expression of TNC by carcinoma cells correlates with the grade of OSCC (Hindermann *et al*, 1999). In our study, TNC expression in tumour cells was not a prognosticator in early stage OTSCC, nor was it a prognosticator among all the stages. On the other hand, the results of the present study indicate that stromal TNC was an independent prognosticator among all stages, and more importantly, among early stages. However, TNC predicted poor prognosis only when detected abundantly in the tumour stroma as there was no difference in prognosis between negative and moderate stromal TNC staining. Taken together, we hypothesise that TNC is related to dissemination of cancer cells and aiding metastasis formation leading to poor prognosis.

Fibronectin is a multifunctional glycoprotein organised in a fibrillary network. It is expressed mainly by fibroblasts, and during development and wound healing. Fibronectin regulates a variety of cell activities such as adhesion, migration, growth and differentiation (Mao and Schwarzbauer, 2005; White *et al*, 2008) and is involved in cancer invasion and metastasis (Stivarou and Patsavoudi, 2015), and stromal FN correlates with poor prognosis or aggressive phenotypes in colorectal cancer (O'Shannessy *et al*, 2014), urothelial carcinoma (Ioachim *et al*, 2005) and breast cancer (Ioachim *et al*, 2002). Fibronectin is one of the markers of EMT, a process essential for dissemination and invasion of carcinoma cells (Chong *et al*, 2012), as well as one of the markers of angiogenesis in primary tumours and metastases (Rybak *et al*, 2007). Fibronectin is upregulated in head and neck cancer (Al Moustafa *et al*, 2002; Ferrari *et al*, 2009), and the presence and amount of FN in tumour stroma is linked to an increased number of cervical metastases, extracapsular spread and elevated risk for disease-specific mortality in OSCCs (Lyons *et al*, 2001). Here, in concordance to previous studies, stromal FN was shown to be an excellent prognosticator among all stages of OTSCC and more importantly among early stage cases. The fact that there were no disease-specific deaths in the stromal FN-negative group among stage I–II patients is itself a remarkable demonstration of the specific nature of this marker. Cellular FN, on the other hand, had prognostic value in the pooled group of all stages in univariate analysis, and additionally it was an independent prognosticator in multivariate analysis. Furthermore, cellular FN predicted survival in stages I–II in Kaplan–Meier, but not in Cox regression uni- or multivariate analysis.

Fibronectin and TNC are co-expressed in several cancers (Hanamura *et al*, 1997; Yoshida *et al*, 1997) including OSCC (Ramos *et al*, 1998) and play a role in the healing by influencing cell adherence and migration especially in epithelial cells (Mackie *et al*, 1988). It has been suggested that both TNC and FN, as antagonists for each other (TNC being an anti-adhesive and FN an adhesive protein), are essential in cancer invasion (Yoshida *et al*, 1997). In support of this, Ramos *et al* (1998) found that TNC matrix production was blocked *in vitro* when FN antibody was introduced to CAF monolayer culture cultivated on FN-coated

glass coverslips. This hypothesis is supported by our results as well, as we observed a strong correlation between stromal TNC and FN in our clinical sample (Supplementary Table 2). Furthermore, additional prognostic value was observed in our cohort among those cases with abundant stromal TNC and FN; among all the stages only one patient survived and no patients among the early stages survived. This implies a strong biological synergy of these proteins in cancer progression.

Our results provide feasible tools for more accurate division of the early stage OTSCC patients into low-risk and high-risk groups. These could be utilised in clinical decision-making in terms of primary neck dissection and multimodality treatments. Furthermore, our results should encourage more basic research on the biology of cancer/TME-interactions, and demonstrate the translational relevance of research on specific proteins. Even though our results provide a basis for using TNC and FN in routine pathology to categorise early stage OTSCC patients into low- and high-risk groups, the cohort was relatively small ($n=98$ for early stage patients). The small number of patients led to relatively broad CIs in Cox regression analysis, especially in abundant stromal FN and TNC among early stage patients. Although we successfully compared our antibodies with commercial ones, these studies should be replicated using other antibodies for TNC and FN, and preferably with larger patient groups.

In conclusion, based on our multi-centre study we recommend the analysis of stromal TNC and FN as an integral part of diagnosis and treatment planning in early stage OTSCC. When stromal TNC and FN both turn out to be negative, elective neck dissection and multimodality treatments should be avoided, whereas abundant stromal staining of these proteins provides a strong signal to consider more aggressive treatments. If future studies demonstrate that immunohistochemical staining of TNC and FN assessed from biopsies is equally specific in the prognostication to that of surgical specimens, the treatment regimen can be chosen preoperatively.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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